[6-2-2]小野文献英訳

# A 13-week oral repeated dose subchronic toxicity study in F344 rats feeding mixed with haematococcus color

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## SUMMARY

A 13-week oral repeated dose toxicity study of haematococcus color, a food additive mainly composed of astaxanthin, was conducted in male and female F344 rats. Rats were randomly divided into 4 groups each consisting of 10 males and 10 females and given CRF-1 powder diet containing 0%, 0.025%, 0.075%, and 0.25% haematococcus color, corresponding to 0%, 0.5%, 1.5%, and 5% as the product. None of the animals died during the administration period. There were no exposure-related changes in body weight gain or food consumptions. Serum biological examinations showed dose-related increase in cholesterol, but the differences were slight and not defined as an adverse effect. No effects related to treatment were noted in hematological examinations and organ weights, and no abnormalities that could be ascribed to exposure to haematococcus color were observed in histopathological examinations. In conclusion, ingestion of haematococcus color in the diet for 13 weeks does not cause any toxicological changes in F344 rats.

Keywords : food additive, haematococcus color, astaxanthin, F344 rats, subchronic toxicity study

## INTRODUCTION

Recently, the demand for food additives derived from natural origin (natural additives) has tended to increase due to the restrictions on use of synthetic additives or to consumers' inclination toward naturals. Although most of the natural additives or their origins have been used as foods based on their long history, they have not been examined their safety scientifically based on the animal studies.

In accordance with partial revisions of Food Sanitation Law and Nutrition Improvement Law in 1995, a newly developed food additive, even a natural food additive, should have an approval to include in Designated List for Food Additives. However, the natural food additives conventionally used have been approved to continue their using hereafter with listing in Name List for Existing Food Additives but without obligation to conduct their toxicity studies [1].

Haematococcus color mainly composed of astaxanthin is an orange ~ red color and has been utilized for a coloring agent mainly in fish or meat-paste products or in oil and fat products. Astaxanthin is a major component of red colors found in marine foods such as

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sea breams, shrimps and crabs etc. and has keenly been expected its utility as a natural food color due to its excellency in color tone and in stability against heating. Furthermore, astaxanthin, a family substance of $\beta$-carotene, has recently been reported possessing excellent potencies such as radical oxygen quenching action [2,3,4], immunoactivating action [5,6] and inhibiting action against cancers [7,8] and come again into the limelight on its use not only for a food color additive but also for its unique function. In the past, astaxanthin was extracted from *Crustacea* such as krill and American crawfishes etc. However, such astaxanthin has not been put into practical use because of various problems in its quality such as poor homogeneity in color tone and offensive smell specialized in its raw material or of economical problems. On the other hand, *Haematococcus pluvialis*, a kind of micro-algae conventionally well known which produces high levels of astaxanthin, has recently been put into practical utilization as a food additive color of astaxanthin extracts obtained by the modified methods of cultivation and extraction [9]. At the moment, the above mentioned micro-alga is scarcely used for a food additive in our country, but in near future, increase of its use as a natural color possessing excellent properties such as color tone and functionality will be much expected.

For haematococcus color, neither report nor information concerning toxicity study or its toxicity has been issued until today. Furthermore, its raw material has not also been supplied as a food. In this regard, it may be necessary to examine its toxicity as soon as possible. At this time, we performed a 13-week subchronic toxicity study of *Haematococcus pluvialis* color in rats, and will report it as follows:

## MATERIALS AND TEST METHOD

### 1. Test substance and administration method

Test substance, haematococcus color used was supplied by Yaegaki Fermentation Co. (Himeji). *Haematococcus pluvialis* color is obtained from *Haematococcus C.A.* AGARCH in such manner : After drying whole algae of AGARCH, grind and extract it with ethanol hydrate, acetone, hexane or two of them or more solvents and vaporize away such solvents and obtain haematococcus color extract which appears a dark brown paste-like liquid with special offensive smell. Haematococcus color is almost insoluble in water, but soluble in ether, acetone, chloroform and benzene and has no hygroscopicity. Further, it is easily discolored by irradiation with U-V and visible rays although excellent in heat resistance. Haematococcus color product is composed of soybean oil diluted to 5% of the color. In this study, pure raw substance of *Haematococcus pluvialis* color (100%) was used in order to avoid effects of the substrate, soybean oil. The test substance was administered by feeding with mixed powder diet (as a Test diet), because purpose of its use is as a food additive. Preparation of Test diets: Dissolve haematococcus color into proper amount of acetone and add them into the basic feed. After removal of acetone by putting them in a draft for one night, mix them with the basic feed to make respective Test diet. Test diets were prepared once per 2 weeks and stored in the feed storage room at 4℃ under light-resistant condition.

### 2. Dose establishment

In this study, doses were established based on the standard volume as a final product

In order to examine the effect of the color as a food additive product, i.e.: the highest dose was set at the dose equivalent to 5% as a product (0.25% of haematococcus color), because the highest dose of food additives in animal study with mixed feeding is recommended as 5% [10]. Prior to conduct this study, a two-week preliminary study was performed with the highest dose of 0.25% haematococcus color in order to assess stability of the color in Test diet and check if the test animals may avoid to take Test diet. Further, a control group with acetone in the same amount in the basic feed was prepared because Test diet is composed of haematococcus color mixed with the basic feed using acetone. In consequence, no differences were observed in any group in body weight gain or feed consumption compared to the control group. Accordingly, in this main study, the doses of haematococcus color were set into four groups as follows: 0.25% as the highest dose (group H, 5% haematococcus color product), 0.075% as the middle dose (group M, 1.5% product), 0.025% as the low dose (group L, 0.5% product) and the control (group C). Although the color in Test diets was discolored easily by irradiation (especially with UV-rays), it was stable stored in the dark place under the condition either at room temperature or in a refrigerator. Therefore, Test diets were kept in feeders with a cover during study.

### 3. Animals and breeding conditions

5-Week aged male and female F344 rats (SPF) were supplied from Japan Charles River (Kanagawa) and tame-bred for 1 week, breeding with the basic feed (Oriental Yeast Co., CRF-1 powder feed). Then, the rats were randomly divided into 4 groups with each male or female rats (10 rats per group) and used for the study. Each 5 male or female rats were housed respectively into transparent plastic cages (width: 26 cm, length: 42 cm, height: 17 cm) which were floored with soft chips (supplied by Sankyo Lab. Service Co. (Tokyo)) and these chips were renewed twice a week. The tap water was freely taken during the test period. The test was performed in the breeding room under the conditions such as room temperature: 24±1℃, R.H: 55±5%, frequency of ventilation: 18 times/hour (all fresh air), lighting and darkening cycle: each 12 hours.

### 4. Examinations and methods

Each 3 groups of male and female rats set for the test groups were received freely the powder diet mixed with respectively prescribed dose of haematococcus color for 13 weeks using powdery feeder of suspending type. Another each one group of male and female rats was fed freely with the basic diet without haematococcus color in the same manner. General signs and incidence of death of the rats were observed every day through the test period and body weight and food consumption were measured every one week. After completion of 13-week feeding, all rats were fasted for one night. Then, the blood was sampled from orbital veins under ether anesthesia and the following tests were conducted by autopsy after bloodletting.

On the sampled blood, erythrocyte count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (Pt), and leukocyte count (WBC) were measured using Sysmex M2000 ( Toa Medical Electronics, Hyogo) in the diluted blood. The WBC typing test was performed using Microx HEG-120A (Omlon, Tokyo) on the blood smear preparation stained by Wright reagent. The serum sample

obtained by centrifugation was used for measurement of such values as total protein (TP, Bullet method), albumin (Alb, BCG method), A/G ratio (calculated from the values of TP and Alb), urea N (BUN, UV method), creatinine (CRN, Enzyme-method), blood sugar (Glc, Enzyme-method), triglyceride (TG, Enzyme-method), total bilirubin (Tbil, Azobilirubin-method), alkaline phosphatase (ALP, Rate-method), transaminase (AT, AsT, Rate-method), calcium (Ca, OCPC-method), inorganic phosphorus (P, molybdic acid direct-method), sodium, potassium, chlorine (Na, K, Cl, ionic electrolyte-method), using Hitachi Model 7150 autoanalyzer.

The organs were ectomized after macroscopic observation. At first, brain, thymus, lungs, heart, spleen, liver, adrenal, kidney and testis (ovaries) were weighed. Then, promptly after the ectomy, skull including nasal cavity, pituitary gland, salivary gland, tongue, trachea, thyroid gland, esophagus, stomach, small intestine, large intestine, pancreas, bladder, prostate, seminal vesicular gland, epididymis, uterus, vagina, mammary gland, lymph node, sternum, femur, skeletal muscle, spinal cord, sciatic nerve, eye ball, skin and muscle were fixed with 10% neutrally buffered formaldehyde solution and after blocking them into paraffin, their micro-slices were prepared and stained using hematoxylin-eosin(H.E.) stain method. Then, histopathological examinations were performed on the control group and the highest dose group.

### 5. Statistical analysis

For body weights, results of hematological and sero-biochemical examinations and absolute and relative weights of organs, F-ratio of each group was analyzed using Bartlett method. One-way layout analysis of variance in homoscedastic case or Kruskal-Wallis test in heteroscedastic case, was performed. When the significant difference was observed between the groups, tests of significance were performed using Dunett method between each group and the control group since they included the same number of animals.

## RESULTS

### 1.General signs

Neither abnormal appearances (skin, body hair) nor effects on general signs were observed in any group through the whole period of test. Furthermore, all tested rats survived up to completion of the study.

### 2.Body weight and food consumption

Fig.1 and 2 show changes of body weight and food consumption of male and female rats during the test period. Further, Table 1 shows mean values of food consumption and intake of test substance. Significant differences between the test groups and the control group of each male and female rats were not observed in body weight and food consumption through the test period. The mean values of food consumption did not differ significantly between the test groups and the control group of each male and female rats during the test period and intake levels of haematococcus color in the test groups correlated approximately dependent to the dose levels.

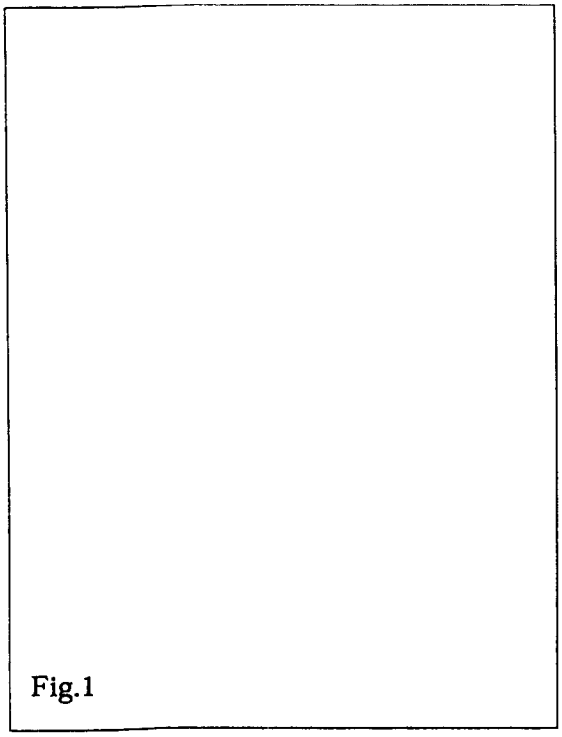

Fig.1

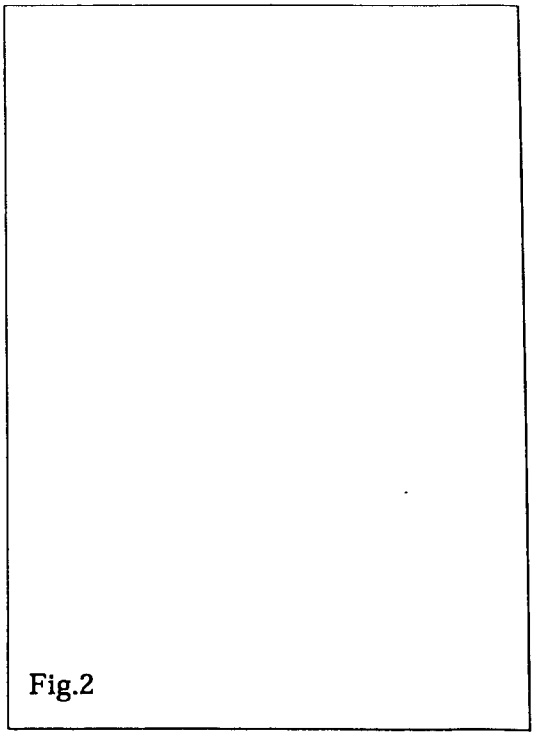

Fig.2

Table 1

3.Hematological examinations

Tables 2, 3 show the results of hematological examinations. In male rats, no significant differences among parameters were observed and any changes affected by dosing of the test substance were not observed in the test groups. The highest dose group of female rats showed significant decrease of MCV and MCH compared to the control group. However, any correlation to the respective dose level was not observed.

4.Sero-biochemical examinations

Tables 4, 5 show the results of sero-biochemical examinations. In the highest dose group (group H) of female rats, significant elevations of T-cho and F-cho levels were

observed. In particular, the elevation of T-cho level correlated significantly to the dose levels of haematococcus color that was also observed in male rats with some tendency but not significant. In male rats, furthermore, significant decreases of A/G ratio, CRN and Na in group H, significant decrease of BUN in group M and significant decrease of CRN and Na in group L were respectively observed but all of them did not correlated to the dose levels significantly and their grade were also very low.

Table 2

Table 3

Table 4

Table 5

5.Organ weight

Tables 6, 7 show the results of organ weights at autopsy. Although a significant decrease of absolute weight of heart was observed only in group M of female rats, any significant differences or changes correlated to the dose levels were not observed in other test groups of male and female rats compared to the control group.

6.Histopathological examinations

Table 8 shows the results of histopathological examinations. No change related to intake of haematococcus color was observed in both male and female rats. Furthermore, it was considered that histopathological lesions found in these examinations seemed to be spontaneously generated lesions which were not induced by dosing of the test substance, taking such grade and incidence into consideration.

## DISCUSSION

A 13-week subchronic toxicity study in F344 rats feeding with mixed test diet was performed in order to assess safety of haematococcus color. A haematococcus color product consists of 5% of *Haematococcus pluvialis color* solved into soybean oil. In this study, however, pure raw substance of *Haematococcus pluvialis* color was used in order to avoid the effect of soybean oil. Test diets were prepared with 5.0, 1.5 and 0.5% preparations for dosing (0.25, 0.075 and 0.025 % of haematococcus color) based on the results of the preliminary study. All male and female rats well grew up through the test period and neither abnormal appearances in skin or body hair nor effects on general signs associated with the test substance were observed. As no differences were observed in body weight and food consumption between the test groups and control group, it was suggested that adding of the color does not affect feeding behaviors or nutritive conditions. In sero-biochemical examinations after completion of 13-week administration, a significant elevation of T-cho was observed in group H of female rats and a small tendency of correlation with dose levels was also observed and that was also observed in male rats. These facts suggest that no toxicological significance existed in this study because the grade of changes seemed small. The cholesterolemic effect of astaxanthin, a main component of *Haematococcus pluvialis*, has been reported[12]. On the other hand, the content of astaxanthin (and its esters) in haematococcus color used in this study shows low as 39% . Furthermore, in the 13-week subchronic toxicity study [13] with *Phaffia* color which contains higher level of astaxanthin than haematococcus color, any change, the same as in our study, was not observed. Therefore, a mechanism for elevating T-cho level in this study cannot be specified. Other changes in hematological examinations or organ weights affected by dosing were not observed. On the other hand, such abnormalities as myocarditis and renal electrolyte-deposition observed in the histopathological examinations in male rats was also observed in the control group that has been reported as spontaneous lesions occurred in F344 rats. Consequently, it suggested that such lesions were not induced by dosing haematococcus color. In the 13-week subchronic toxicity study feeding with *Phaffia* color mainly consisting of astaxanthin, the same as the haematococcus color used in our study, the highest, 5% dose group did not show any toxicity [13]. Haematococcus color used in this

study also did not show any effect on food consumption and body weight gain. Further, any clear toxicological findings in hematological and sero-biochemical examinations, as well as in organ weights and histophathological tests affected by dosing, were not observed. Consequently, it was suggested that toxicity of haematococcus color on rats in this feeding study was negligibly very low.

Table 6

Table 7

Table 8

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6-1

# ヘマトコッカス藻色素のF344ラットによる13週間反復混餌投与毒性試験

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# A 13-week subchronic oral toxicity study of haematococcus color in F344 rats

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A 13-week oral repeated dose toxicity study of haematococcus color, a food additive mainly composed of astaxanthin, was conducted in male and female F344 rats. Rats were randomly divided into 4 groups each consisting of 10 males and 10 females and given CRF-1 powder diet containing 0%, 0.025%, 0.075%, and 0.25% haematococcus color, correspond to 0%, 0.5%, 1.5%, and 5% as the product. None of the animals died during the administration period. There were no exposure-related changes in body weight gain or food consumptions. Serum biochemical examinations showed dose-related increase in cholesterol, but the differences were slight and not defined as an adverse effect. No effects related to treatment were noted in hematological examinations and organ weights, and no abnormalities that could be ascribed to exposure to heamatococcus color were observed in histopathological examinations. In conclusion, ingestion of haematococcus color in the diet for 13 weeks does not cause any toxicological changes in F344 rats.

Keywords: food additive, haematococcus color, astaxanthin, F344 rats, subchronic toxicity study

## 緒　　言

天然物由来の食品添加物（天然添加物）の需要は，近年の合成添加物に対する使用規制や消費者の自然志向などより増大の傾向にある．しかし天然添加物の多くは，それ自体もしくはその原料が，長年食用に供されてきた経験に基づいて使用されてはいるが，その安全性について動物実験などに基づいた科学的な検討の乏しいものが少なくない．平成7年の食品衛生法及び栄養改善法の一部改正より，新たに開発される食品添加物については，天然添加物であっても合成添加物と同様に指定制度の対象となったが，従来使用されてきた天然添加物については既存添加物名簿に収載され毒性試験の実施などを義務付けることなく引き続きその使用が認められている[1]．

ヘマトコッカス藻色素は，アスタキサンテンを主成分とする橙色～赤色色素で，主として魚肉ねり製品や油脂製品などの着色料として使用される．アスタキサンテンは，鯛，海老および蟹など水産物の赤色の主成分をなす物質で，色調，熱安定性にすぐれており天然食用色素としての有用性が期待されてきた．さらに近年，βカロテンの類縁体であるアスタキサンテンが，活性酸素消去能[2,3,4]や免疫賦活能[5,6]および発がん抑制作用[7,8]などを有することが報告され，単に食品用色素としてだけではなくその機能からも再び注目されている．これまでアスタキサンテンは，オキアミやアメリカザリガニなどの甲殻類より抽出されたが，色素濃度の均一性や原料特有の臭気など品質および経済性などの問題から食用色素としての実用化には至っていない．一方，*Haematococcus pluvialis*（ヘマトコッカス藻）はアスタキサンチンを高産生することが従来より知られる微細藻類で，近年，培養法や抽出法の改良により，その抽出色素がアスタキサンテン系食品着色料として実用化された[9]．現在のところ我が国では，食品添加用としてほとんど使用されてはいないが，優れた色調と機能性を有する天然着色料として，今後の使用増加が予想される．

ヘマトコッカス藻色素についてはこれまで毒性試験および毒性に関しての報告はない．また原料のヘマトコッカス藻が食用に供されることもないため，早急に毒性の検討が必要であると考えられる．そこで今回，ラットを用いてヘマトコッカス藻色素混餌投与による13週間の亜慢性毒性試験を実施したので報告する．

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## 実験材料および方法

### 1. 被験物質および投与方法

ヘマトコッカス藻色素はヤエガキ発酵株式会社（姫路）より供与されたものを用いた．ヘマトコッカス藻色素は，コナヒゲムシ科ヘマトコッカス（*Haematococcus CA. AGARCH*）の全藻を乾燥後，粉砕し，含水エタノール，アセトン，ヘキサンもしくはこれらを2種類以上混合したもので抽出し溶媒を除去したものであり，特有の臭気を有する暗褐色のペースト状液体である．ヘマトコッカス藻色素は，水にはほとんど溶けず．エーテル，アセトン，クロロホルム，ベンゼンなどに溶解する．吸湿性はなく，耐熱性に優れるが，紫外線および可視光線により容易に退色する．ヘマトコッカス藻色素製剤は大豆油にヘマトコッカス藻色素を5%となるよう溶解したものである．本試験では，添加剤である大豆油の影響を除外するため，ヘマトコッカス藻色素原体（100%）を用い，投与は被験物質の使用目的が食品添加物であることから粉末飼料による混餌により行った．色素添加飼料の調整は，ヘマトコッカス藻色素を適量のアセトンに溶解して基礎飼料に添加し，一晩ドラフト内でアセトンを除去した後，基礎飼料と混合してそれぞれ規定量の添加飼料とした．添加飼料の調整は1回/2週行い，調整した添加飼料は4℃に維持された飼料保管室に遮光して保存した．

### 2. 投与用量

本試験では食品添加物製剤としての影響を調べることを目的としたため，用量設定は製剤としての量を基準に行った．すなわち食品添加物の混餌投与試験における上限が5%とされている[10]ことから，製剤として5%に相当する量（0.25%ヘマトコッカス藻色素）を上限とした．本試験を行うにあたり，添加飼料中の色素安定性および動物の被験物質混合飼料の忌避を確認する目的で，0.25%ヘマトコッカス藻色素を最高用量とする2週間の予備試験を行った．またヘマトコッカス藻色素をアセトンに溶解して飼料に混じるため，同量のアセトンを添加した対照群を設けた．この結果，いずれの群においても体重，摂餌量に基礎飼料対照群とのあいだに差は認められなかった．これらより本試験におけるヘマトコッカス藻色素の最高用量は，0.25%（H群，ヘマトコッカス藻色素製剤 5%）とし，以下，0.075%（M群，同 1.5%），0.025%（L群，同 0.5%）および対照群（C群）を加えた4群で試験を行った．また，添加飼料中の色素は，光（特に紫外光）により容易に退色するが，暗所においては室温および冷蔵のいずれの条件下においても安定であった．試験期間中は，給餌器に蓋をして遮光した．

### 3. 動物および飼育条件

5週齢のF344ラット（SPF）雌雄を日本チャールス・リバー（神奈川）より購入し，基礎飼料（オリエンタル酵母（株）CRF-1粉末飼料）で1週間馴化飼育した後，無作為に雌雄各4群（1群10匹）に分け，それぞれを試験に供した．動物は雌雄とも透明なプラスチックケージ（幅 26cm，長さ 42cm，高さ 17cm）に5匹ずつ収容し，床敷はソフトチップ（三協ラボサービス社（東京））を用い，週2回交換を行った．試験期間中，飲料水として水道水を自由に摂取させた．試験は室温 24±1℃，湿度 55±5%，換気回数 18回/時（オールフレッシュ），12時間明暗サイクルの条件下の飼育室にて行った．

### 4. 検査項目および方法

雌雄各3群を被験物質投与群とし，それぞれ規定量のヘマトコッカス藻色素を混合した粉末飼料を13週間，懸架式粉末給餌器を用いて自由に摂取させた．対照群として雌雄各1群にはヘマトコッカス藻色素を含まない基礎飼料を同期間自由に摂取させた．試験期間中，一般状態および死亡動物の有無を連日観察し，体重および摂餌量の測定を週1回行った．投与開始13週後に全動物を一晩絶食させた後，エーテル麻酔下に眼窩静脈叢より採血し，放血後剖検し以下に示す検査を行った．

採取した血液について，希釈血液を用いてSysmex M2000（東亜医用電子，兵庫）により赤血球数（RBC），ヘモグロビン量（Hb），ヘマトクリット値（Ht），平均赤血球容積（MCV），平均赤血球血色素量（MCH），平均赤血球血色素濃度（MCHC），血小板数（Plt），及び白血球数（WBC）を測定した．またライト染色を施した血液塗抹標本を作成し白血球の型別分類を，Microx HEG-120A（オムロン，東京）を用いて測定した．遠心分離により得た血清試料を用いて，総蛋白量（TP，ビュレット法），アルブミン量（Alb，BCG法），アルブミン・グロブリン比（TP及びAlbより算出），尿素窒素（BUN，UV法），クレアチニン量（CRN，酵素法），血糖値（Glc，酵素法），トリグリセライド量（TG，酵素法），総コレステロール量（T-Cho，酵素法），遊離コレステロール（F-Cho，酵素法），総ビリルビン（Tbil，アゾビリルビン法），アルカリフォスファターゼ活性値（ALP，Rate法），トランスアミナーゼ活性値（A T，AsT，Rate法），γ-グルタミルトランスフェラーゼ（γ-GT，Rate法），カルシウム（Ca，OCPC法），無機リン（P，モリブデン酸直接法），ナトリウム，カリウム，クロール（Na，K，Cl，イオン電解質法）を日立7150型オートアナライザーを用いて測定した．

諸臓器は肉眼的に観察した後摘出し，脳，胸腺，肺，心臓，脾臓，肝臓，副腎，腎臓および精巣（卵巣）については重量を測定した後に，鼻腔を含む頭蓋，下垂体，唾液腺，舌，気管，甲状腺，食道，胃，小腸，大腸，膵臓，膀胱，前立腺，精嚢腺，精巣上体，子宮，膣，乳腺，リンパ節，胸骨，大腿骨，骨格筋，脊髄，坐骨神経，眼球，皮膚およ

び筋肉等については摘出後直ちに10%中性緩衝ホルマリン液にて固定し，常法に従いパラフィン包埋後，薄切片を作製し，ヘマトキシリン・エオジン（H.E.）染色を施し，対照群と最高用量群について病理組織学的検索を行った．

5. 統計学的処理[11]

体重，血液学的・血清生化学的検査結果および臓器の絶対重量と相対重量については，各群の分散比をBartlettの方法で検定し，等分散の場合は一元配置の分散分析を，不等分散の場合はKruscal-Wallisの方法により検定を行った．群間に有意差が認められた場合は各群の例数が等しいことより，Dunnett法により対照群と各群との有意差検定を行った．

## 結　果

1. 一般状態

試験期間を通して，いずれの群においても外見的（皮膚，体毛など）異常および被験物質投与に伴う一般状態への影響は認められなかった．また，すべての動物が試験終了時まで生存した．

2. 体重および摂餌量

試験期間中の雌雄ラットの体重および摂餌量の推移をそれぞれFig.1，Fig.2に示した．また，試験期間中の平均摂餌量および被験物質摂取量をTable 1に示した．雌雄各群ともに体重，摂餌量に試験期間を通して対照群との間に大きな差は認められなかった．また試験期間中の平均摂餌量においても，雌雄とも対照群と被験物質投与群との間に大きな差はみられず，被験物質であるヘマトコッカス藻色素の摂取量も被験物質の用量段階にほぼ相関した．

3. 血液形態学的検査

血液学的検査の結果をTable 2,3に示した．雄ではいずれの項目においても有意差および被験物質投与による変化は認められなかった．雌のH群で対照群に比べMCV,MCHの低下が有意に認められたが，いずれも投与用量との相関は認められなかった．

4. 血清生化学的検査

血清生化学的検査の結果をTable 4,5に示した．雌では，T-ChoおよびF-Choの上昇がH群で有意に認められた．特にT-Choの上昇はヘマトコッカス藻色素投与用量に相関しており，雄においても有意ではないが同様の傾向が認められた．雄ではこのほか，H群でA/G比,CRNおよびNaの減少，M群でBUNの減少，L群でCRNおよびNaの減少が有意に認められたが，いずれも用量に相関するものではなくまたその程度もわずかであった．

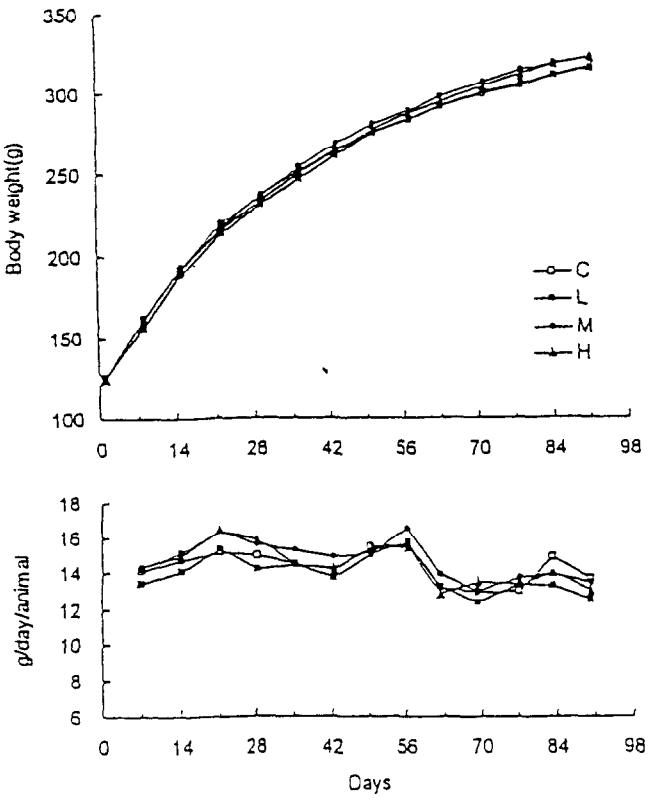


Fig.1. Body weight and food consumption curves of male F344 rats fed diet containing haematococcus color for 13 weeks; Dose levels of haematococcus color were C: 0 %, L: 0.025 %, M: 0.075 %, H: 0.25 %, respectively.

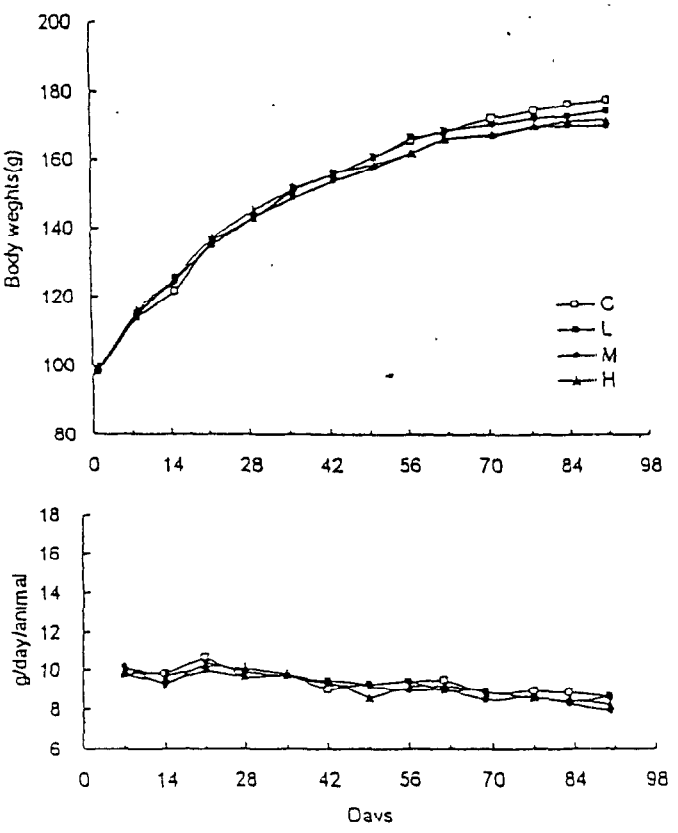


Fig.2. Body weight and food consumption curves of female F344 rats fed diet containing haematococcus color for 13 weeks; Dose levels of haematococcus color were C. 0 %, L: 0.025 %, M: 0.075 %, H: 0.25 %, respectively.

Table 1. Mean food consumption and haematococcus color intake in F344 rats

| Sex | Group | No.of rats examined | Food consumption | Mean daily intakes of Food | Mean daily intakes of Haematococcus color | Total intakes of haematococcus |
|---|---|---|---|---|---|---|
| | | | g/rat/day | g/kg/day | mg/kg/day | mg/kg/13 weeks |
| Male | C | 10 | 14.2 | 60.9 | 0.0 | 0.0 |
| | L | 10 | 13.9 | 60.0 | 15.0 | 1364.0 |
| | M | 10 | 14.6 | 61.7 | 46.3 | 4208.9 |
| | H | 10 | 14.4 | 61.3 | 153.2 | 13941.1 |
| Female | C | 10 | 9.4 | 65.1 | 0.0 | 0.0 |
| | L | 10 | 9.3 | 64.1 | 16.0 | 1457.5 |
| | M | 10 | 9.2 | 64.0 | 48.0 | 4367.3 |
| | H | 10 | 9.5 | 66.1 | 165.2 | 15034.1 |

5. 臓器重量

剖検時の臓器重量の結果をTable 6,7に示した．雌のM群で心臓の実重量の減少が有意に認められたほかには，対照群と比較して有意差もしくは用量相関性のある変化は雌雄ともに認められなかった．

6. 病理組織学的検査

病理組織学的検索の結果をTable 8に示した．雌雄ともにヘマトコッカス藻色素の摂取に依存すると思われる変化は認められなかった．また病理組織学的検査で散見された病変はいずれも既に自然発生病変として報告されているもので，その程度，頻度を考慮に入れるとこれら病変が被験物質投与により誘発されたものではないと考えられた．

## 考　　察

ヘマトコッカス藻色素の安全性を確認するため，F344ラットの混餌投与による13週間の亜慢性毒性試験を実施した．ヘマトコッカス藻色素製剤はヘマトコッカス藻色素原体を大豆油に5%となるよう溶解したものであるが，本試験では大豆油による影響を除くためヘマトコッカス藻色素原体を用い，投与用量は予備試験の結果などから製剤として5.0，1.5および0.5%（ヘマトコッカス藻色素として0.25，0.075および0.025%）とした．試験期間を通じて，雌雄各群ともに順調に成育し被験物質投与に伴う皮膚，体毛など外見上の異常や一般状態への影響は認められなかった．体重や摂餌量に対照群と投与群で差は認められず，本色素の添加は摂餌行動や栄養状態に特に影響を及ぼさないことが示された．13週投与後の血清生化学検査において，雌のH群でT-choの上昇が有意に認められ，弱いながらも用量相関性が認められ，また雄においても同様の傾向が認められたが，変化の程度も軽度であることから毒性学的意義は乏しいものと考察された．ヘマトコッカス藻の主成分であるアスタキサンチンについてはコレステロール増加作用[12]が報告されている．一方，本試験で用いたヘマトコッカス藻色素のアスタキサンチン（及びエステル体）含有量は約39%（data not shown）と低く，またアスタキサンチンを主成分としてヘマトコッカス藻色素よりも高濃度に含有するファフィア色素の13週間の亜慢性毒性試験[13]においては，同様の変化は認められていないことから本試験におけるT-cho上昇の機序は不明である．この他，血液形態学的検査や臓器重量には投与による変化は認められず，病理組織学的検査において認められた雄の心筋炎および腎臓の鉱質沈着等はいずれも対照群においても発生し，またF344ラットでの自然発生病変として報告されているものであることなどからヘマトコッカス藻色素の投与により誘発されたものではないと考察された．本色素と同様にアスタキサンチンを主成分とするファフィア色素では，F344ラットへの13週間混餌投与により最高5.0%で毒性は示されていない．[13] 今回のヘマトコッカス藻色素についても，いずれの投与群においても摂餌量および体重増加への影響はなく，また血液形態学的および血清生化学検査，臓器重量および病理組織学的にも投与に伴う明らかな毒性所見は認められなかったことから，ラットでの混餌投与によるヘマトコッカス藻色素の毒性は極めて低いものと考察された．

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Table 2. Hematological data of F344 male rats fed diet containing haematococcus color for 13 weeks

| Group | | C | L | M | H |
|---|---|---|---|---|---|
| % of haematococcus color | | 0.000 | 0.025 | 0.075 | 0.250 |
| No. of animals | | 10 | 10 | 10 | 10 |
| RBC | $10^{12}$/l | 9.55 ± 0.31 | 9.68 ± 0.22 | 9.67 ± 0.18 | 9.68 ± 0.20 |
| Hb | g/dl | 16.1 ± 0.3 | 16.1 ± 0.3 | 16 ± 0.2 | 16.1 ± 0.3 |
| Ht | % | 45.3 ± 1.6 | 45.9 ± 1 | 45.8 ± 0.8 | 45.7 ± 1.1 |
| MCV | fl | 47.5 ± 0.4 | 47.4 ± 0.4 | 47.3 ± 0.5 | 47.2 ± 0.4 |
| MCH | pg | 16.8 ± 0.6 | 16.6 ± 0.1 | 16.6 ± 0.3 | 16.6 ± 0.2 |
| MCHC | g/dl | 35.5 ± 1.4 | 35.1 ± 0.3 | 35.1 ± 0.4 | 35.2 ± 0.4 |
| Plt | $10^{12}$/l | 0.54 ± 0.11 | 0.51 ± 0.07 | 0.5 ± 0.08 | 0.49 ± 0.13 |
| WBC | $10^{3}$/l | 6.51 ± 1.11 | 6.78 ± 0.89 | 6.61 ± 1.1 | 7.16 ± 1.3 |
| Neut-B | % | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Base | % | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Eosino | % | 0.8 ± 0.7 | 1.1 ± 0.8 | 1 ± 0.7 | 1 ± 0.5 |
| Neut-S | % | 16.4 ± 3.1 | 16.4 ± 5.6 | 17.5 ± 4.1 | 19.4 ± 6.1 |
| Lympho | % | 81 ± 4.1 | 80.3 ± 5.5 | 78.9 ± 2.9 | 77.1 ± 7 |
| Mono | % | 1.9 ± 1.4 | 2.2 ± 0.9 | 2.7 ± 1.3 | 2.5 ± 1.4 |

Values represent mean ± S.D..

Table 3. Hematological data of F344 female rats fed diet containing haematococcus color for 13 weeks

| Group | | C | L | M | H | |
|---|---|---|---|---|---|---|
| % of haematococcus color | | 0.000 | 0.025 | 0.075 | 0.250 | |
| No. of animals | | 10 | 10 | 10 | 10 | |
| RBC | $10^{12}$/l | 8.88 ± 0.26 | 8.93 ± 0.28 | 8.95 ± 8.17 | 8.93 ± 0.18 | |
| Hb | g/dl | 15.8 ± 0.4 | 15.8 ± 0.5 | 15.8 ± 0.3 | 15.7 ± 0.3 | |
| Ht | % | 44.2 ± 1.3 | 44.3 ± 1.3 | 44.4 ± 0.8 | 44.1 ± 1.0 | |
| MCV | fl | 49.8 ± 0.3 | 49.6 ± 0.2 | 49.6 ± 0.3 | 49.3 ± 0.2 | ** |
| MCH | pg | 17.8 ± 0.1 | 17.7 ± 0.1 | 17.7 ± 0.2 | 17.5 ± 0.1 | ** |
| MCHC | g/dl | 35.7 ± 0.3 | 35.6 ± 0.3 | 35.6 ± 0.3 | 35.6 ± 0.2 | |
| Plt | $10^{12}$/l | 0.48 ± 0.12 | 0.47 ± 0.12 | 0.47 ± 0.05 | 0.48 ± 0.09 | |
| WBC | $10^{9}$/l | 5.49 ± 0.72 | 4.62 ± 0.68 | 5.35 ± 1.01 | 5.16 ± 0.89 | |
| Neut-B | % | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | |
| Base | % | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | |
| Eosino | % | 1.3 ± 0.7 | 1.4 ± 1.0 | 1.1 ± 1.1 | 1.1 ± 0.6 | |
| Neut-S | % | 19.0 ± 3.7 | 18.0 ± 4.0 | 16.3 ± 4.0 | 16.0 ± 3.4 | |
| Lympho | % | 77.4 ± 3.8 | 79.3 ± 4.2 | 80.7 ± 4.0 | 81.2 ± 3.5 | |
| Mono | % | 2.3 ± 1.3 | 1.3 ± 0.9 | 2.0 ± 0.8 | 1.7 ± 0.8 | |

Values represent mean ± S.D..

** show significant difference from the control groups at $p<0.01$.

Table 4. Serum biochemical data of F344 male rats fed diet containing haematococcus color for 13 weeks

| Group | | C | L | M | H |
|---|---|---|---|---|---|
| % of haematococcus color | | 0.000 | 0.025 | 0.075 | 0.250 |
| No. of animals | | 10 | 10 | 10 | 10 |
| TP | g/dl | 6.13 ± 0.13 | 6.14 ± 0.16 | 6.19 ± 0.08 | 6.15 ± 0.14 |
| Alb | g/dl | 3.92 ± 0.12 | 3.93 ± 0.08 | 3.92 ± 0.05 | 3.85 ± 0.07 |
| A/G | | 1.77 ± 0.09 | 1.78 ± 0.06 | 1.73 ± 0.05 | 1.68 ± 0.06 * |
| BUN | mg/dl | 21.4 ± 1.6 | 20.8 ± 0.8 | 19.8 ± 1.1 * | 20.2 ± 1.2 |
| CRN | mg/dl | 0.37 ± 0.03 | 0.35 ± 0.04 | 0.35 ± 0.01 | 0.33 ± 0.01 ** |
| Glc | mg/dl | 119 ± 12 | 120 ± 6 | 119 ± 8 | 121 ± 9 |
| TG | mg/dl | 133 ± 37 | 142 ± 38 | 128 ± 37 | 139 ± 24 |
| TCho | mg/dl | 64 ± 5 | 64 ± 5 | 65 ± 6 | 68 ± 3 |
| FCho | mg/dl | 11.9 ± 1.3 | 11.9 ± 1.7 | 11.5 ± 1.5 | 12.3 ± 0.8 |
| TBil | mg/dl | 0.04 ± 8.8 | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.05 ± 0.02 |
| AlP | mU/ml | 228 ± 20 | 227 ± 21 | 218 ± 11 | 218 ± 15 |
| AlT | mU/ml | 57 ± 5 | 58 ± 10 | 55 ± 4 | 59 ± 4 |
| AsT | mU/ml | 88 ± 18 | 84 ± 15 | 84 ± 12 | 90 ± 13 |
| γ-GT | mU/ml | 0.22 ± 0.29 | 0.17 ± 0.3 | 0.12 ± 0.15 | 0.18 ± 0.17 |
| Ca | mg/dl | 10.3 ± 0.1 | 10.5 ± 0.2 | 10.4 ± 0.1 | 10.4 ± 0.1 |
| P | mg/dl | 5.8 ± 0.4 | 5.6 ± 0.5 | 5.7 ± 0.4 | 5.5 ± 0.6 |
| Na | mEq/l | 139 ± 2 | 139 ± 1 | 139 ± 1 | 138 ± 1 |
| K | mEq/l | 4.9 ± 0.4 | 4.8 ± 0.3 | 5 ± 0.3 | 4.9 ± 0.3 |
| Cl | mEq/l | 101 ± 4 | 102 ± 1 | 101 ± 2 | 101 ± 2 |

Values represent mean ± S.D..

* and ** show significant difference from the control groups at $p<0.05$ and $p<0.01$, respectively

Table 5. Serum biochemical data of F344 female rats fed diet containing haematococcus color for 13 weeks

| Group | | C | L | M | H |
|---|---|---|---|---|---|
| % of haematococcus color | | 0.000 | 0.025 | 0.075 | 0.250 |
| No. of animals | | 10 | 10 | 10 | 10 |
| TP | g/dl | 6.04 ± 0.13 | 5.96 ± 0.15 | 6.05 ± 0.14 | 5.93 ± 0.11 |
| Alb | g/dl | 4.01 ± 0.11 | 3.96 ± 0.1 | 4.05 ± 0.11 | 4.01 ± 0.08 |
| A/G | | 1.99 ± 0.12 | 1.98 ± 0.1 | 2.03 ± 0.09 | 2.09 ± 0.07 |
| BUN | mg/dl | 18.1 ± 1.5 | 19.1 ± 1.2 | 19 ± 1.5 | 17.7 ± 1.4 |
| CRN | mg/dl | 0.35 ± 0.02 | 0.36 ± 0.02 | 0.36 ± 0.02 | 0.34 ± 0.02 |
| Glc | mg/dl | 110 ± 7 | 113 ± 7 | 112 ± 7 | 109 ± 10 |
| TG | mg/dl | 83 ± 20 | 80 ± 16 | 76 ± 18 | 82 ± 24 |
| TCho | mg/dl | 91 ± 6 | 92 ± 10 | 98 ± 8 | 107 ± 6 ** |
| FCho | mg/dl | 20.2 ± 1.3 | 19.7 ± 2.6 | 21.5 ± 2.4 | 22.8 ± 1.6 * |
| TBil | mg/dl | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.05 ± 0.01 | 0 07 ± 0.02 |
| AlP | mU/ml | 138 ± 11 | 153 ± 11 | 143 ± 15 | 147 ± 14 |
| AlT | mU/ml | 40 ± 3 | 39 ± 2 | 40 ± 3 | 40 ± 4 |
| AsT | mU/ml | 83 ± 10 | 81 ± 11 | 85 ± 10 | 78 ± 6 |
| γ-GT | mU/ml | 0.32 ± 0.32 | 0.13 ± 0.16 | 0.10 ± 0.14 | 0.37 ± 0.40 |
| Ca | mg/dl | 10.1 ± 0.1 | 10.2 ± 0 2 | 10.3 ± 0.2 | 10.3 ± 0.2 |
| P | mg/dl | 4 0 ± 0.6 | 4.6 ± 0.6 | 4.5 ± 0.3 | 4 5 ± 0.7 |
| Na | mEq/l | 138 ± 1 | 137 ± 1 | 137 ± 1 | 138 ± 1 |
| K | mEq/l | 4 6 ± 0.3 | 4.5 ± 0.2 | 4.7 ± 0.3 | 4 8 ± 0.2 |
| Cl | mEq/l | 102 ± 1 | 101 ± 1 | 102 ± 1 | 102 ± 1 |

Values represent mean ± S.D..

* and ** show significant difference from the control groups at $p<0.05$ and $p<0.01$, respectively

Table 6. Organ weights of F344 male rats fed diet containing haematococcus color for 13 weeks

| Group | | C | L | M | H |
|---|---|---|---|---|---|
| % of haematococcus color | | 0.000 | 0.025 | 0.075 | 0.250 |
| No. of animals | | 10 | 10 | 10 | 10 |
| Absolute | | | | | |
| Body Weights | g | 310.65 ± 9.34 | 308.24 ± 10.15 | 315.69 ± 10.8 | 315.48 ± 11.12 |
| Brain | g | 1.931 ± 0.051 | 1.92 ± 0.062 | 1.932 ± 0.059 | 1.917 ± 0.048 |
| Heart | g | 0.884 ± 0.034 | 0.899 ± 0.059 | 0.894 ± 0.057 | 0.909 ± 0.054 |
| Lungs | g | 0.967 ± 0.056 | 0.923 ± 0.047 | 0.933 ± 0.035 | 0.941 ± 0.029 |
| Liver | g | 7.12 ± 0.419 | 7.088 ± 0.288 | 7.308 ± 0.376 | 7.15 ± 0.227 |
| Kidney | g | 1.732 ± 0.064 | 1.725 ± 0.089 | 1.791 ± 0.087 | 1.779 ± 0.061 |
| Spleen | g | 0.571 ± 0.026 | 0.573 ± 0.021 | 0.569 ± 0.029 | 0.559 ± 0.021 |
| Testes | g | 3.003 ± 0.079 | 2.945 ± 0.119 | 2.943 ± 0.081 | 2.943 ± 0.102 |
| Adrenal | mg | 32.41 ± 3.49 | 32.25 ± 4.12 | 34.21 ± 3.22 | 33.52 ± 2.83 |
| Thymus | mg | 204.51 ± 54.71 | 202.08 ± 46.27 | 185.95 ± 40.44 | 199.16 ± 38.71 |
| Relative | | | | | |
| Brain | g% | 0.622 ± 0.025 | 0.622 ± 0.023 | 0.613 ± 0.028 | 0.608 ± 0.019 |
| Heart | g% | 0.283 ± 0.011 | 0.29 ± 0.018 | 0.284 ± 0.017 | 0.287 ± 0.014 |
| Lungs | g% | 0.311 ± 0.02 | 0.299 ± 0.014 | 0.294 ± 0.011 | 0.299 ± 0.011 |
| Liver | g% | 2.29 ± 0.087 | 2.299 ± 0.062 | 2.315 ± 0.068 | 2.267 ± 0.06 |
| Kidney | g% | 0.558 ± 0.016 | 0.559 ± 0.016 | 0.566 ± 0.021 | 0.564 ± 0.022 |
| Spleen | g% | 0.182 ± 0.01 | 0.186 ± 0.007 | 0.179 ± 0.009 | 0.177 ± 0.008 |
| Testes | g% | 0.967 ± 0.038 | 0.954 ± 0.039 | 0.933 ± 0.046 | 0.934 ± 0.037 |
| Adrenal | mg% | 10.45 ± 1.24 | 10.48 ± 1.39 | 10.84 ± 1.12 | 10.64 ± 1.03 |
| Thymus | mg% | 65.62 ± 16.44 | 65.26 ± 13.08 | 59.02 ± 13.66 | 63.28 ± 13.29 |

Values represent mean ± S.D.

Table 7. Organ weights of F344 female rats fed diet containing haematococcus color for 13 weeks

| Group | | C | L | M | H |
|---|---|---|---|---|---|
| % of haematococcus color | | 0.000 | 0.025 | 0.075 | 0.250 |
| No. of animals | | 10 | 10 | 10 | 10 |
| Absolute | | | | | |
| Body Weights | g | 172.19 ± 5.46 | 170.53 ± 7.06 | 165.33 ± 9.01 | 166.86 ± 4.06 |
| Brain | g | 1.78 ± 0.02 | 1.787 ± 0.037 | 1.769 ± 0.029 | 1.782 ± 0.036 |
| Heart | g | 0.567 ± 0.029 | 0.548 ± 0.018 | 0.532 ± 0.024 ** | 0.555 ± 0.028 |
| Lungs | g | 0.71 ± 0.044 | 0.7 ± 0.042 | 0.676 ± 0.034 | 0.675 ± 0.046 |
| Liver | g | 3.658 ± 0.205 | 3.735 ± 0.229 | 3.612 ± 0.175 | 3.708 ± 0.184 |
| Kidney | g | 1.049 ± 0.062 | 1.063 ± 0.069 | 1.032 ± 0.055 | 1.054 ± 0.044 |
| Spleen | g | 0.370 ± 0.02 | 0.367 ± 0.026 | 0.355 ± 0.021 | 0.347 ± 0.018 |
| Ovaries | g | 48.72 ± 11.41 | 48.31 ± 8.44 | 49.19 ± 7.78 | 49.72 ± 5.24 |
| Adrenal | mg | 38.32 ± 4.67 | 39.44 ± 4.83 | 35.50 ± 4.41 | 37.64 ± 4.51 |
| Thymus | mg | 167.11 ± 24.53 | 168.28 ± 25.31 | 164.76 ± 16.85 | 152.67 ± 23.88 |
| Relative | | | | | |
| Brain | g% | 1.035 ± 0.04 | 1.047 ± 0.034 | 1.072 ± 0.052 | 1.069 ± 0.039 |
| Heart | g% | 0.329 ± 0.014 | 0.322 ± 0.014 | 0.323 ± 0.013 | 0.333 ± 0.018 |
| Lungs | g% | 0.412 ± 0.026 | 0.411 ± 0.03 | 0.409 ± 0.023 | 0.406 ± 0.034 |
| Liver | g% | 2.128 ± 0.119 | 2.192 ± 0.121 | 2.187 ± 0.10 | 2.223 ± 0.12 |
| Kidney | g% | 0.609 ± 0.036 | 0.624 ± 0.037 | 0.624 ± 0.027 | 0.633 ± 0.031 |
| Spleen | g% | 0.214 ± 0.01 | 0.215 ± 0.011 | 0.215 ± 0.018 | 0.206 ± 0.012 |
| Ovaries | g% | 28.34 ± 6.68 | 28.22 ± 4.18 | 29.83 ± 5.00 | 29.81 ± 3.25 |
| Adrenal | mg% | 22.24 ± 2.47 | 23.18 ± 3.14 | 21.49 ± 2.62 | 22.54 ± 2.53 |
| Thymus | mg% | 97.02 ± 13.75 | 98.57 ± 13.37 | 99.69 ± 9.05 | 91.71 ± 15.46 |

Values represent mean ± S.D.

** show significant difference from the control groups at $p<0.01$.

Table 8. Histological findings in male and female rats fed diet containing haematococcus color for 13 weeks

| Sex | Male | | Female | |
|---|---|---|---|---|
| Group | C | H | C | H |
| No. of animals examined | 10 | 10 | 10 | 10 |
| Heart | | | | |
| Myocardial degeneration | 1 | 2 | 0 | 0 |
| Intramyocardial cell infiltration | 1 | 0 | 0 | 0 |
| Lung | | | | |
| Microgranuloma | 1 | 0 | 0 | 1 |
| Liver | | | | |
| Exratmedullary hematopoiesis | 1 | 0 | 0 | 0 |
| Microgranuloma | 1 | 4 | 3 | 1 |
| Vacuolation of hepatocyte | 0 | 1 | 0 | 0 |
| Kidney | | | | |
| Tubular regeneration | 2 | 2 | 0 | 0 |
| Hyalin droplet | 10 | 10 | 0 | 0 |
| Calcification of collecting tubule | 1 | 0 | 0 | 0 |
| Calcification at cortico-medullary junction | 0 | 0 | 0 | 1 |
| Hyalin cast | 0 | 1 | 0 | 0 |
| Pancreas | | | | |
| Vacuolation of acinar cell | 1 | 0 | 0 | 1 |
| Spleen | | | | |
| Microgranuloma | 0 | 0 | 1 | 0 |
| Adrenal gland | | | | |
| Accessory adrenal gland | 1 | 1 | 0 | 1 |
| Dilatation of sinusoid | 0 | 0 | 1 | 1 |


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